Arsenic Induces Tumor Necrosis Factor α Release and Tumor Necrosis Factor Receptor 1 Signaling in T Helper Cell Apoptosis

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Long-term exposure to arsenic induces arsenical cancers in human beings. Arsenic has been shown to induce apoptosis in a variety of cell systems. Previous studies revealed that patients with arsenic-induced Bowen's disease showed a defective cell-mediated immunity and decreased percentages of T cell and T helper cell subpopulations in peripheral mononuclear cells. The purpose of this study was to investigate the effects of arsenic on T cell survival and function in mononuclear cells. Arsenic concentrations higher than 1 μM induced tumor necrosis factor α release from mononuclear cells and caused a cytotoxic effect on T cells. When exposed to higher concentrations of arsenic, apoptosis was induced. CD4+ cells were the major apoptoic population in mononuclear cells. Tumor necrosis factor receptor 1 expression on CD4+ cells, but not Fas/FasL, was significantly enhanced by arsenic treatment compared to other mononuclear cells. Increased expressions of tumor necrosis factor receptor 1 related death domain proteins and activated caspases were observed. These findings indicate that tumor necrosis factor receptor 1 signaling is the major pathway in arsenic-induced T helper cell apoptosis. ***Key words: cytokine/ cytokine receptor/ cytotoxicity/ lymphocytes/ sodium arsenite. J Invest Dermatol 119:812–819, 2002***

Arsenic is a well-established human carcinogen. Epidemiologic studies demonstrated that long-term exposure to inorganic arsenic through ingestion and inhalation is associated with an increased risk of malignant tumors developing in many organs, such as the lung, stomach, liver, colon, kidney, and urinary bladder, in addition to skin cancers (Chen *et al*, 1985). *In vitro* studies have demonstrated that arsenic induces genetic and epigenetic effects (Chen *et al*, 1997; Yih *et al*, 1997) as well as apoptosis in a variety of cell systems. Arsenite is considered to be the most likely carcinogenic form of arsenic (Rossman, 1998). Arsenite induces chromosome aberrations, aneuploudy, and micronuclei (Rossman, 1999). Arsenite at nontoxic concentrations was found to act as a comutagen with other agents. The comutagenic effects of arsenite are probably the result of inhibition of DNA repair. Arsenite inhibits the repair of DNA damage induced by X-rays and ultraviolet (UV) radiation (Synder *et al*, 1989) and postreplication repair of UV-induced damage (Lee-Chen *et al*, 1992). Furthermore, arsenite potentiates X-ray and UV-induced chromosomal damage in peripheral human lymphocytes and fibroblasts (Jha *et al*, 1992).

A defective cell-mediated immune function is noticed in patients with arsenical skin cancers. Impaired delayed-type hypersensitivity response to 2,4-dinitrochlorobenzene, reduction in T cell and T helper cell percentages, and a decrease in mitogenic responses of peripheral mononuclear cells (MNCs) were found in arsenic-induced Bowen's disease. The defective cell-mediated immune function in Bowen's disease is due to impairment of membrane interleukin-2 (IL-2) receptor expression in lymphocytes after chronic arsenic exposure (Yu *et al*, 1998). Arsenic induced biphasic effects on lymphocyte mitogenesis. There was an amplification of response at lower concentrations and an inhibition of mitogenesis at higher concentrations of arsenic (McCabe *et al*, 1983; Yu *et al*, 1992). Arsenic has been shown to induce apoptosis in leukemic cells (Chen *et al*, 1996; Wang *et al*, 1998; Zhang *et al*, 1998), plasma cell lines and myeloma cells (Rousselot *et al*, 1999), esophageal carcinoma cells (Shen *et al*, 1999), immortalized human cervical epithelial cells (Zheng *et al*, 1999), and umbilical vein endothelial cells (Roboz *et al*, 2000). One of the important mechanisms of apoptosis is regulated by death receptor associated death domain signaling intermediates, which contain receptor activation induced binding of death domain proteins, such as tumor necrosis factor (TNF) receptor-associated death domain protein (TRADD) and Fas-associated death domain protein (FADD), and further activation of caspase cascades that initiate apoptotic death (Wallach *et al*, 1999). A major apoptotic signal in MNCs is mediated by TNF-α and its receptor. Signaling by TNF receptor causes the association of an adapter protein TRADD with the intracellular death domain of the TNF receptor 1 (TNF-R1) molecule (Hsu *et al*, 1996; Ashkenazi and Dixit, 1998). TRADD mediates the subsequent recruitment of an adapter protein FADD to form a death-inducing signaling complex, which initiates apoptosis through activation of caspase cascades. The purpose of this study was to investigate the effects of arsenic on human peripheral MNC functions and T cell apoptosis. We found that arsenic stimulated TNF-α release from

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Abbreviations: FADD, Fas-associated death domain protein; MNC, mononuclear cell; PARP, poly ADP ribose polymerase; TNF-R1, tumor necrosis factor receptor 1; TRADD, TNF receptor-associated death domain protein.

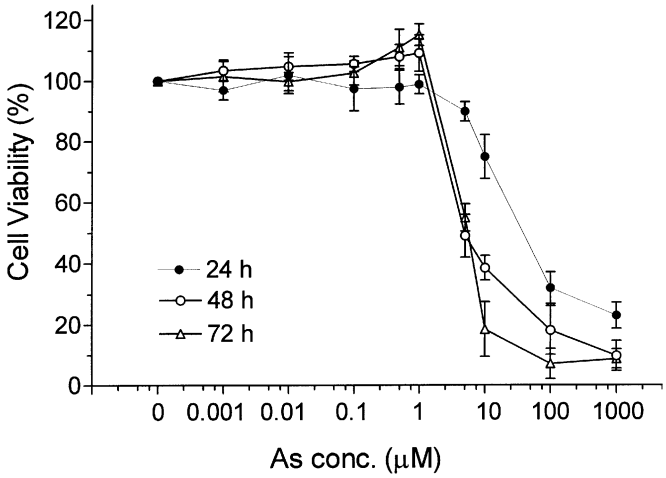

Figure 1. Effects of arsenic on MNC viability. Freshly obtained normal human MNCs were seeded (10^6 cells per ml) in 96-well microplates, treated with different concentrations of sodium arsenite (in PBS), and incubated in a CO_2 incubator at 37°C for 24 h, 48 h or 72 h. The cell viability was analyzed by XTT assay. Cell viability was rapidly decreased with arsenic exposures higher than 1 μM and about 50% of the MNCs died when exposed to 5 μM of arsenic for 48 h and 72 h. Viability of the control group (no arsenic added; PBS only) after 24 h, 48 h or 72 h incubation was taken as 100% (mean ± SD; $n = 18$).

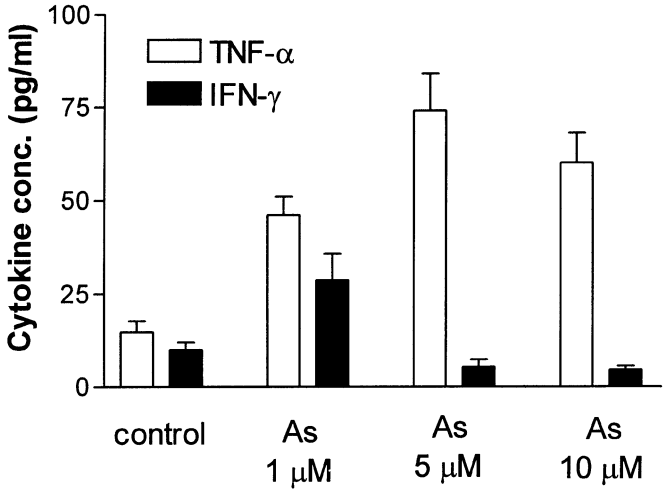

Figure 2. Effects of arsenic on TNF-α and IFN-γ release from MNCs. After 48 h of 0 (control), 1, 5, and 10 μM of arsenic exposure, the MNC supernatant (per 10^6 cells) was collected and the concentrations of cytokines were measured with commercialized ELISA kits. The *white bars* are concentrations of TNF-α; IFN-γ is shown as *black bars* (pg per ml). Both IFN-γ and TNF-α were increased with exposure to 1 μM of arsenic; at 5 μM, IFN-γ release was inhibited but the release of TNF-α was further stimulated. All data in the ELISA test were measured by linear regression standard curves in which r^2 should larger than 0.99 (mean ± SD; $n = 12$).

MNCs and TNF-R1 signaling plays a major role in arsenic-induced T helper cell apoptosis.

MATERIALS AND METHODS

Isolation of MNCs and arsenic treatment Venous blood was collected from 12 healthy adults (25–40 y old). MNCs were isolated from heparinized venous blood of these donors after centrifugation at 300 × *g* for 20 min over a Ficoll-Hypaque cushion (specific gravity 1.077) as reported previously (Yu *et al*, 1989). Freshly obtained MNCs were suspended in RPMI-1640 medium (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum and adjusted to a concentration of 1.0×10^6 cells per ml. Sodium arsenite (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) was then added to final concentrations of 0.01, 0.1, 0.5, 1, 5, or 10 μM. Arsenic-treated MNCs were cultured at 37°C in a humidified incubator with 5% CO_2 atmosphere. The following experiments were performed in triplicate.

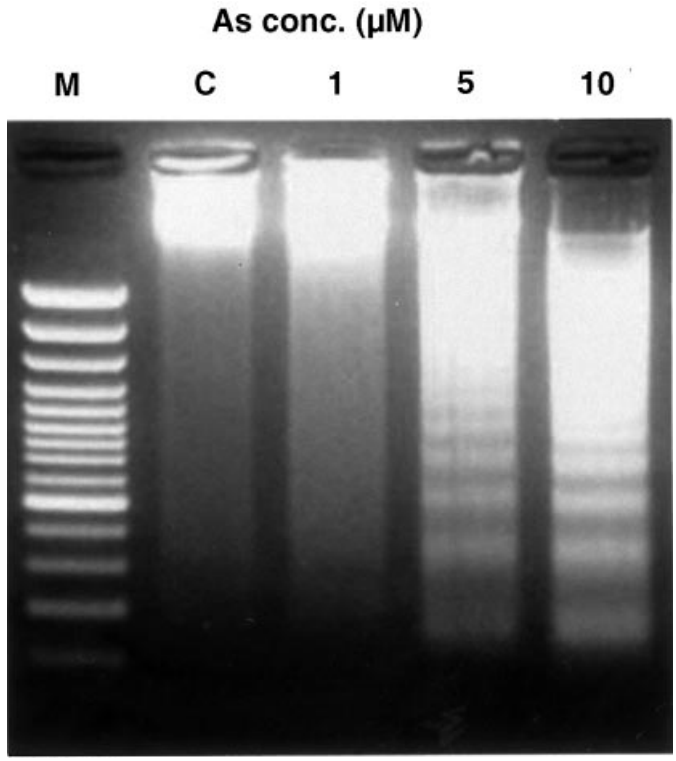

Figure 3. Agarose gel electrophoresis analysis of apoptotic DNA fragments. Cultured MNCs were exposed to arsenic for 48 h, and then cellular DNA was isolated. Total DNA from MNC samples was loaded into a 2% agarose gel for electrophoresis at 50 V for 1 h. 180 bp DNA ladders were observed as a typical feature for apoptosis. At 5 and 10 μM of arsenic treatment, DNA patterns showed a typical feature of apoptotic DNA ladders in agarose gel (M, 100 bp DNA marker; C, control).

Cell viability assay The commercially available kit for cell proliferation (XTT assay kit; Roche, Mannheim, Germany) was used according to the manufacturer's instruction. Freshly prepared MNCs were seeded into 96-well plates (1.0×10^6 cells per ml) and various concentrations of sodium arsenite were added in triplicate. After 24, 48, or 72 h of treatment, the XTT reagents were then added into each well and incubated at 37°C for 4 h to generate colorimetric formazan products. Absorbance of formazan product was measured at a wavelength of 450 nm with a reference wavelength of 630 nm (Jost *et al*, 1992). The results were expressed as relative cell viability.

Measurement of cytokines in culture supernatants by enzyme-linked immunosorbent assay (ELISA) After 48 h of arsenic treatment, cultured MNCs were centrifuged at 2000 rpm for 10 min and the cell-free supernatants were obtained for cytokine measurement. TNF-α and interferon-γ (IFN-γ) were determined using commercially available ELISA kits (Quantikine, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

DNA laddering analysis for apoptosis The DNA fragmentation analysis (DNA ladder) was assessed by agarose gel electrophoresis according to Enari *et al* (1998) with a slight modification. Arsenic-treated or control MNCs (2×10^6 cells) were collected and centrifuged at 1200 rpm for 5 min and then resuspended in a lysis buffer [50 mM Tris–HCl pH 8.0, 5 mM ethylenediamine tetraacetic acid (EDTA), 1.2% sodium dodecyl sulfate, 150 mM NaCl, 0.2 mg per ml proteinase K] followed by incubation at 37°C overnight. Cellular DNA was isolated by phenol extraction and the DNA samples were carefully loaded into the wells of a 2.0% agarose gel. Electrophoresis was carried out in TAE

Table I. Effects of arsenic on MNCs

Treatment	Percentage of cell populations			
	CD3+ (T cells)	CD19+ (B cells)	CD3+/CD4+ (T helper)	CD3+/CD8+ (T cytotoxic)
Effects of arsenic on CD3 and CD19 populations in MNCs[a]				
Control	75.1 ± 2.8	19.3 ± 1.6		
As 1 μM	77.4 ± 4.3	17.8 ± 2.5		
As 5 μM	61.7 ± 3.2[c]	18.8 ± 2.7		
Effects of arsenic on CD4+ and CD8+ T cells (CD3+, CD4, or CD8 single positive) subpopulations in MNCs[b]				
Control			40.5 ± 4.2	36.1 ± 2.4
As 1 μM			38.4 ± 4.1	37.8 ± 3.1
As 5 μM			28.6 ± 3.7[c]	35.4 ± 2.0

[a]Forty-eight hour incubated control cells 1 μM or 5 μM arsenic exposed cells were double stained with CD3-PE and CD19-FITC. Percentages of cell populations were measured by flow cytometry. A decrease in CD3+ cell percentage was noticed in the 5 μM arsenic-treated group.
[b]Cells were double stained with CD3-PE/CD4-FITC or CD3-PE/CD8-FITC. Percentages of cell populations were measured by flow cytometry. A decrease in T helper cell percentage was noticed in the 5 μM arsenic-treated group.
[c]$p < 0.05$ (mean ±SD; $n = 12$).

Table II. Flow cytometric analysis of apoptotic MNC subpopulations affected by arsenic[a]

Treatment	Percentage of apoptotic cells (TUNEL+)		
	CD4+	CD8+	CD19+
Control	3.5 ± 1.3	2.8 ± 1.0	3.3 ± 1.4
As 1 μM	4.8 ± 1.7	4.2 ± 1.4	2.8 ± 1.2
As 5 μM	12.6 ± 0.8[b]	5.4 ± 1.1	4.1 ± 1.8

[a]Cultured MNC with or without arsenic exposure for 48 h and double stained with TUNEL/CD4, TUNEL/CD8, or TUNEL/CD19. The data showed CD4+ cells were the major apoptotic subpopulation in MNC by arsenic.
[b]$p < 0.05$ (mean ±SD; $n = 12$).

Table III. Flow cytometric analysis of TNF-R1 expression in T cell subpopulations[a]

	TNF-R1 expression (mean fluorescence intensity)	
	CD4+	CD8+
Control	35.9 ± 1.5	31.0 ± 0.8
As 1 μM	36.2 ± 1.2	32.3 ± 1.7
As 5 μM	44.1 ± 2.1[b]	29.7 ± 1.6

[a]After 48 h incubation, MNCs were double stained with TNF-R1/CD4 or TNF-R1/CD8. Mean fluorescence intensity of TNF-R1 was analyzed. Arsenic enhanced TNF-RI expression on CD4+ cells but not on CD8+ cells.
[b]$p < 0.05$ (mean ±SD; $n = 12$).

buffer at 50 V for 1 h and the DNA was visualized by ethidium bromide staining.

Flow cytometric analysis of MNC populations, apoptotic subpopulations, and apoptosis-related receptors Populations of lymphocytes in MNCs were determined by flow cytometric analysis. Briefly, arsenic-treated and control MNCs were stained with CD3-PE, CD19-FITC, CD4-FITC, or CD8-FITC antibodies (Becton Dickinson, San Jose, CA) in an ice-bath for 30 min and then fixed with 4% paraformaldehyde (in PBS), and the percentages of each population were measured by flow cytometry (ESP, Couter, Miami, FL) (Yu *et al*, 1998).

To distinguish the apoptotic cells from MNC populations, a TUNEL kit (Roche, Mannheim, Germany) was used. Arsenic-treated and control MNCs were stained with phycoerythrin (PE) conjugated antibodies against CD4, CD8, or CD19 (Becton Dickinson) and fixed with 4% paraformaldehyde. After washing twice with PBS, the cells were incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Permeabilized cells were washed with PBS and 50 μl of TUNEL reaction mixture (contains terminal deoxynucleotidyl transferase and nucleotide mixture) was added to each sample. A 60 min reaction at 37°C was required to label the DNA strand breaks with fluorescein isothiocyanate (FITC). The CD4-PE or CD8-PE/TUNEL-FITC double stained MNCs were washed and percentages of positively stained cells were analyzed by flow cytometry.

The flow cytometric method was also used in the determination of TNF-R1, TNF-R2, and Fas/FasL expression on MNCs. MNCs were collected and stained with FITC-conjugated monoclonal anti-TNF-R1, anti-TNF-R2, anti-Fas, or anti-FasL antibodies. After washing twice with PBS, CD3-PE, CD19-PE, CD4-PE, or CD8-PE were then stained. The double stained MNCs were washed with PBS, fixed with 4% paraformaldehyde (in PBS), and analyzed by flow cytometry.

TNF receptor blocking test Freshly isolated MNCs (10^7 cells) were suspended in 0.45 ml culture medium, and 50 μl of polyclonal anti-TNF receptor antibody (1 mg per ml; PeproTech, Rocky Hill, NJ) was added. After 30 min incubation at 37°C, the MNC–antibody solution was diluted with 9.5 ml of culture medium to adjust to 1.0×10^6 cells per ml MNCs and 5 μg per ml of antibody concentration. This antibody-containing cell suspension was then transferred into 96-well plates and cultured at different arsenic concentrations. After 48 h culture, cell viability was measured by XTT assay.

Western blotting analysis of apoptosis-associated proteins For sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting of FADD, TRADD, and caspases, total cellular protein extract from MNCs was obtained by lysing the cells in ice-cold lysis buffer (50 mM Tris, 5 mM EDTA, 0.1% Triton X-100, 150 mM NaCl, and mixed cocktail of protease inhibitors). After 12,000 rpm centrifugation to remove cell debris, supernatants were collected and the protein concentrations were measured by protein quantification kit (Bio-Rad, Hercules, CA). 40 μg total protein of each sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (using 12.5% acrylamide gels). Proteins on polyacrylamide gels were then transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked in 2% skim milk/PBS with 0.5% Tween-20 for 1 h at room temperature and incubated with primary and secondary antibodies. Polyclonal antibodies against human TRADD, FADD, caspase 8, caspase 3, and poly ADP ribose polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA) were used at a dilution of 1:500 in the blocking solution. The chemiluminescence substrate kit (Pierce, Rockford, IL) was used to visualize the presence of specific proteins in the nitrocellulose membrane.

Statistical analysis All values are presented as mean ± SD. The comparisons of lymphocyte populations and subpopulations among control and test groups were assessed using the unpaired Student's *t* test. A p-value of < 0.05 was considered as statistically significant.

RESULTS

Arsenic revealed cytotoxicity in MNCs Freshly prepared MNCs were exposed to various concentrations of sodium arsenite and incubated for 24 h, 48 h, and 72 h at 37°C. Arsenic concentrations lower than 1 μM showed no significant effect on

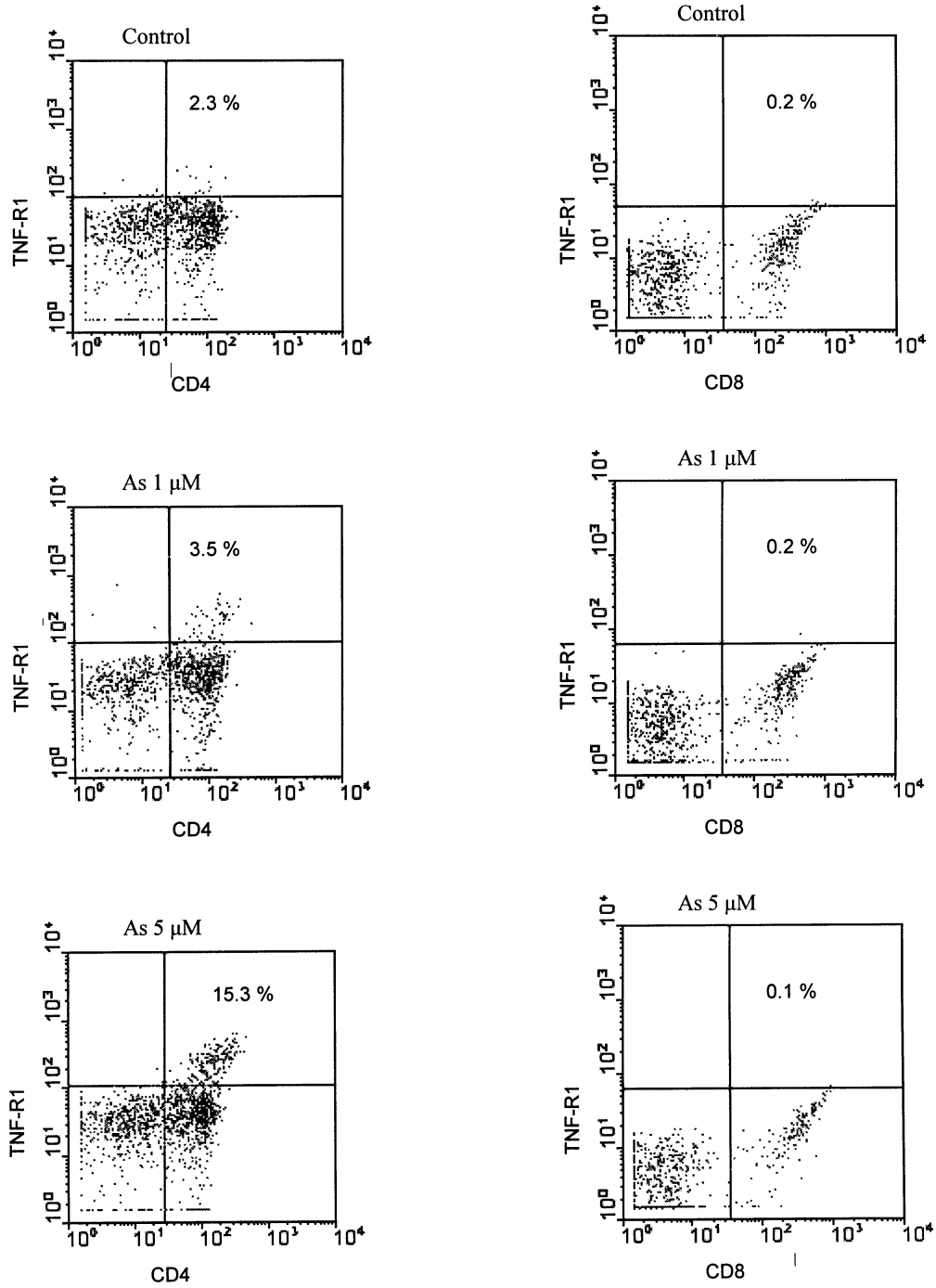

Figure 4. Flow cytometric analysis of TNF-R1+ percentage in T cell subpopulations. After 48 h incubation, MNCs were double stained with TNF-R1/CD4 or TNF-R1/CD8 and percentages of TNF-R1+ cells were analyzed. Arsenic enhanced TNF-RI expression on CD4+ cells but not on CD8+ cells. Arsenic 5 μM stimulated a significant increase in TNF-R1 expression on CD4+ cells.

Table IV. Apoptosis-associated receptors expressed on CD4+ cells by flow cytometry[a]

	Fas	FasL	TNF-R1	TNF-R2
Control	2.2% ± 1.8%	0.9% ± 0.7%	2.1% ± 1.2%	1.6% ± 0.3%
As 1 μM	4.5% ± 2.4%	1.1% ± 0.2%	3.2% ± 1.4%	1.7% ± 0.5%
As 5 μM	7.1% ± 5.3%	0.1% ± 0.1%	15.2% ± 2.7%[b]	0.7% ± 0.2%

[a]After 48 h incubation, MNC were double stained with CD4 and each receptor/ligand. All data in this table are for CD4+ cells and the percentage of expression in each receptor/ligand was compared with the control group. TNF-R1 is the only significant apoptosis-related receptor expressed on CD4+ cells by 5 μM arsenic.

[b]$p < 0.05$ (mean ±SD; $n = 12$).

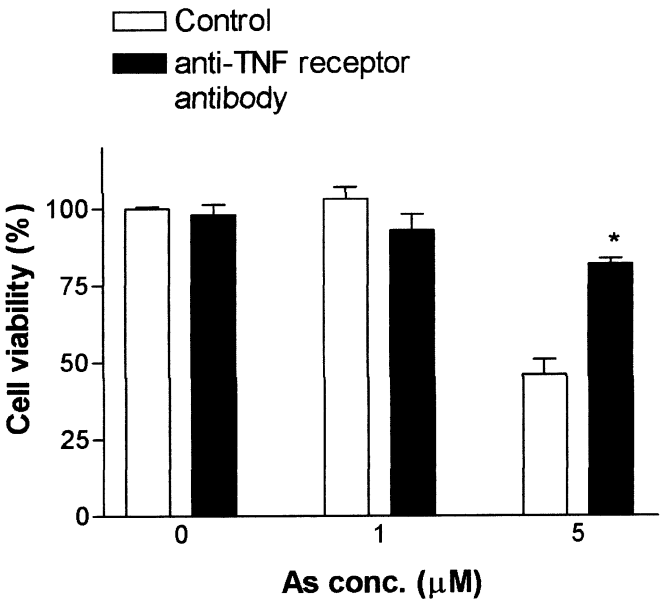

Figure 5. TNF receptor blocking test for arsenic-induced cytotoxicity. Anti-TNF receptor antibody or control antibody treated MNCs were cultured under 0, 1, 5, and 5 μM of arsenic exposure for 48 h. The cell viability was analyzed by XTT assay. Anti-TNF receptor antibody revealed that arsenic-induced cytotoxicity at 5 μM arsenic in MNCs was inhibited (*cell viability of anti-TNF receptor antibody *versus* control antibody treated MNCs at the same arsenic concentration $p < 0.05$; mean ± SD; $n = 12$).

MNC survival by XTT assay. At 1 μM arsenic, about 10% increase in cell viability was noticed both at 48 h and 72 h incubation, but not at 24 h. Cell viability rapidly decreased when arsenic concentrations were higher than 1 μM. At 48 h and 72 h incubation, arsenic concentrations lower than 1 μM revealed a similar pattern in cell viability. 50% cell viability was noticed at 5 μM arsenic at both 48 h and 72 h incubation (**Fig 1**). Therefore, in this study we chose 0, 1, and 5 μM arsenic and 48 h incubation for the apoptosis assay. This concentration-dependent effect of arsenic on MNC viability was also confirmed by trypan blue exclusion and BrdU incorporation assays (data not shown).

Arsenic induced TNF-α release from MNCs Effects of arsenic on the release of proinflammatory cytokines, i.e., TNF-α and IFN-γ, were studied by ELISA. The results showed that TNF-α and IFN-γ were significantly stimulated by arsenic. As shown in **Fig 2**, both IFN-γ release (28.8 ± 7.1 pg per ml) and TNF-α release (46.1 ± 5.0 pg per ml) were increased by exposure to 1 μM of arsenic. In contrast, at 5 μM, IFN-γ release was inhibited but the release of TNF-α was further stimulated (74.2 ± 9.8 pg per ml). These results indicated that TNF-α might play an important role in arsenic-induced cell death.

Arsenic induced MNC apoptosis In order to clarify the type of cell death induced by arsenic, an agarose gel electrophoresis was performed. We found that 5 and 10 μM of arsenic were able to induce DNA fragmentation, which showed a typical feature of apoptotic DNA ladders in agarose gel (**Fig 3**). In parallel, TUNEL staining was performed and revealed the same results (data not shown).

Arsenic induced CD4+ cell apoptosis To identify the cell populations that were affected by arsenic, percentages of cell populations in MNCs were analyzed using flow cytometry. CD3 and CD19 were used as markers to detect T cells and B cells, respectively. As shown in **Table I(*a*)**, CD3+ cells were significantly decreased by 5 μM arsenic treatment. We further analyzed the subpopulations in CD3+ cells using CD4 (T helper) and CD8 (T cytotoxic) markers. The results indicated that CD4+ cells were the target cells of arsenic (**Table I*b***). The TUNEL/CD4, TUNEL/CD8, and TUNEL/CD19 double staining method was then used to detect apoptotic populations in MNCs. As shown in **Table II,** CD4+ cells were the major apoptotic subpopulation induced by arsenic.

Arsenic stimulated a significant increase in TNF-R1 expression on CD4+ cells In order to further confirm if the TNF pathway was involved in arsenic-induced apoptosis, we studied TNF-R1 expressions on MNC populations. Arsenic enhanced TNF-R1 expression on CD3+ cells but not other cell populations, i.e., CD19+ B cells and CD14+ monocytes, in MNCs (data not shown). Furthermore, arsenic enhanced both TNF-R1 expression intensity (**Table III**) and percentage (**Fig 4**) on CD4+ cells, but not on CD8+ cells. Other apoptosis-associated receptors, such as Fas/FasL and TNF-R2, were also assayed and revealed no significant effects (**Table IV**). This result was confirmed by a TNF receptor inhibition assay. When TNF-R1 was blocked by a polyclonal antibody, arsenic-induced cytotoxicity in MNCs was inhibited (**Fig 5**), and TUNEL+ apoptotic CD4+ cells were decreased (**Fig 6**). These results indicated that TNF-R1 is the major apoptosis-related receptor expressed on CD4+ cells by arsenic treatment.

Arsenic induced TNF-R1 signaling in MNCs TNF-R1-associated apoptotic factors and caspases were detected by Western blotting. The death domain proteins TRADD and FADD were increased by arsenic treatment; in addition, the active forms of caspase-8 and caspase-3 were also increased (**Fig 7**). Activated caspase-3 can cleave its substrate PARP to 85 kDa and 16 kDa fragments. In this study, at higher concentrations of arsenic, an 85 kDa fragment cleaved from PARP was observed (**Fig 7**). These results indicated that TNF-R1 apoptotic signaling played a significant role in arsenic-induced CD4+ cell death.

DISCUSSION

Patients with arsenic-induced Bowen's disease showed a defective cell-mediated immunity and decreased percentages of T cell and T helper cell subpopulations in peripheral MNCs (Yu *et al*, 1992; 1998). In this study, concentrations higher than 1 μM revealed a toxic effect on MNCs. Furthermore, higher concentrations of arsenic induced T cell apoptosis with a significant decrease in the percentage of CD4+ T cells. This result is consistent with the finding of a reduction in T helper cell percentage in arsenic-induced Bowen's disease (Yu *et al*, 1998). CD4+ cells were the major apoptotic population in T cells. Arsenic at 1 μM stimulates both TNF-α and IFN-γ release from MNCs. TNF-α is mainly released from monocytes/macrophages (Vassalli, 1992), and IFN-γ is produced by T helper cell type 1 (Constant and Bottomly, 1997). Arsenic concentrations of 5 μM and 10 μM induce significant toxic effects including an apoptotic effect on T helper cells; therefore IFN-γ release is suppressed. On the other hand, monocytes/macrophages are relatively resistant to arsenic and TNF-α production is still stimulated by 5 μM and 10 μM concentrations of arsenic. A slight but significant increase in TNF-α release between 1 μM and 5 μM is noticed. In contrast, a dramatic increase in cell death exists between these two concentrations. Clearly, TNF-α is not the only explanation for apoptosis induced by arsenic. A concentration of 5 μM arsenic induces a significantly higher percentage of TNF-R1-expressed CD4+ cells compared to 1 μM arsenic. In addition, 5 μM arsenic significantly increases the mean fluorescence intensity level in CD4+ cells. These findings induced by 5 μM arsenic provide a reasonable explanation for the significantly higher percentage of CD4+ cell apoptosis compared to 1 μM arsenic. Furthermore, the TNF receptor blocking test revealed an antiapoptotic effect of 5 μM arsenic. Pretreatment by anti-TNF-R1 antibody was able to inhibit arsenic-induced cytotoxicity in MNCs and apoptosis in CD4+ cells. There is a

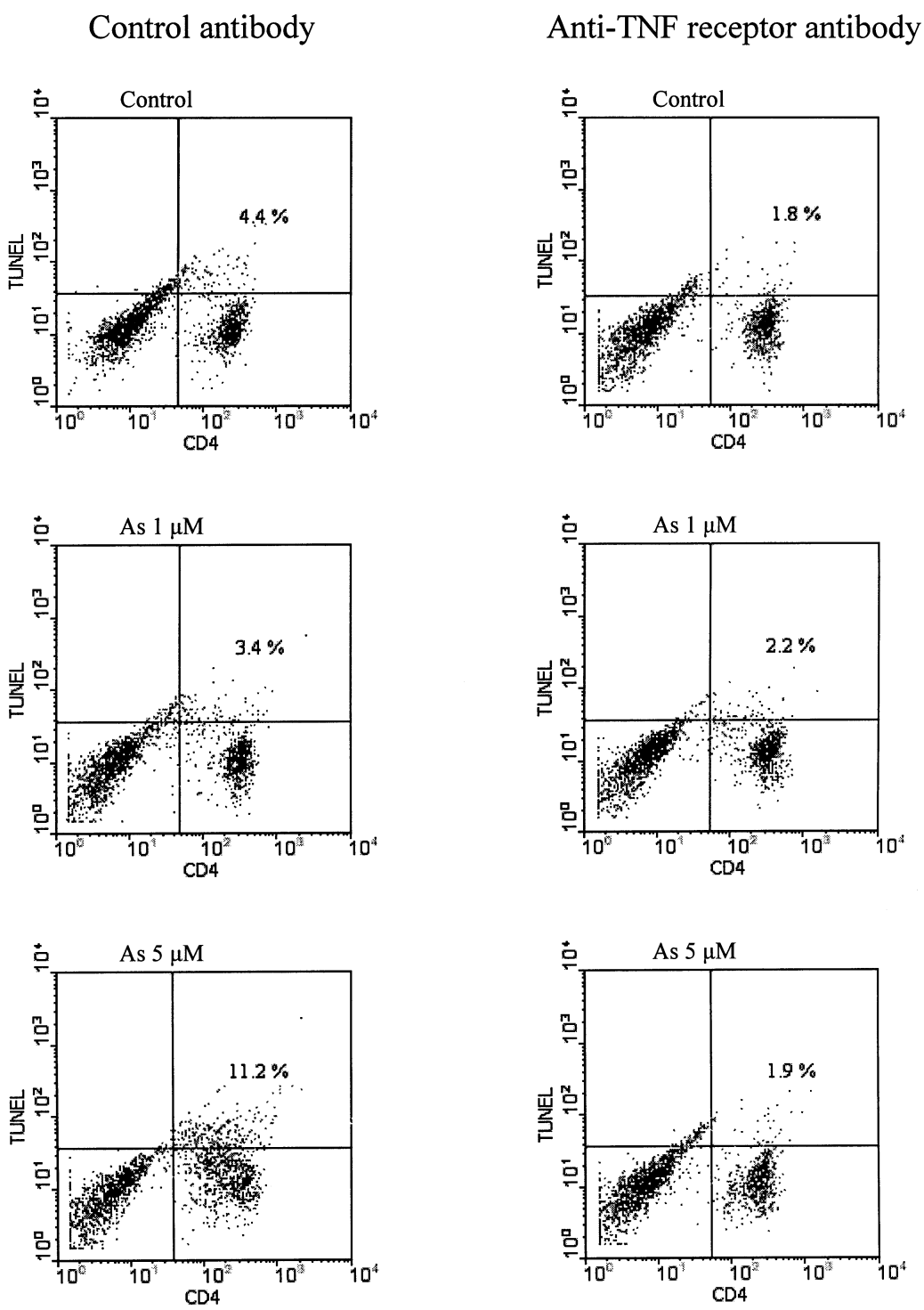

Figure 6. TNF receptor blocking test for arsenic-induced CD4+ cell apoptosis. Anti-TNF receptor antibody or control antibody treated MNCs were exposed to arsenic for 48 h. Then MNCs were double stained with TUNEL/CD4 and analyzed by flow cytometry. The percentage of TUNEL+/CD4+ cells is shown to compare the number of apoptotic CD4+ populations with different treatments. The TNF receptor blocking test revealed an antiapoptotic effect on CD4+ cells at 5 μM arsenic.

significant decrease in both spontaneous and inducible TNF-α and IFN-γ production in MNCs in patients with arsenic-induced Bowen's disease. This impaired cellular immunity is due to defective IL-2 receptor expression induced by low-dose and chronic exposure to arsenic (Yu *et al*, 1998). The different release patterns of TNF-α and IFN-γ between arsenic-induced Bowen's disease and this study can be explained by different concentrations and exposure periods of arsenic.

Active T cell apoptosis takes place indirectly by the antigen-induced expression of death cytokines, chiefly TNF and FasL/Apo-1 L (Russell *et al*, 1991; Brunner *et al*, 1995; Zheng *et al*, 1995; Nagata, 1997). Activation of the TNF with two distinct surface receptors, TNF-R1 and TNF-R2 has been reported (Tartaglia and Goeddel, 1992; Smith *et al*, 1994). Signaling by TNF-α is initiated by binding to TNF-R1, which causes the association of death domain proteins TRADD, FADD, etc. to form a death-inducing

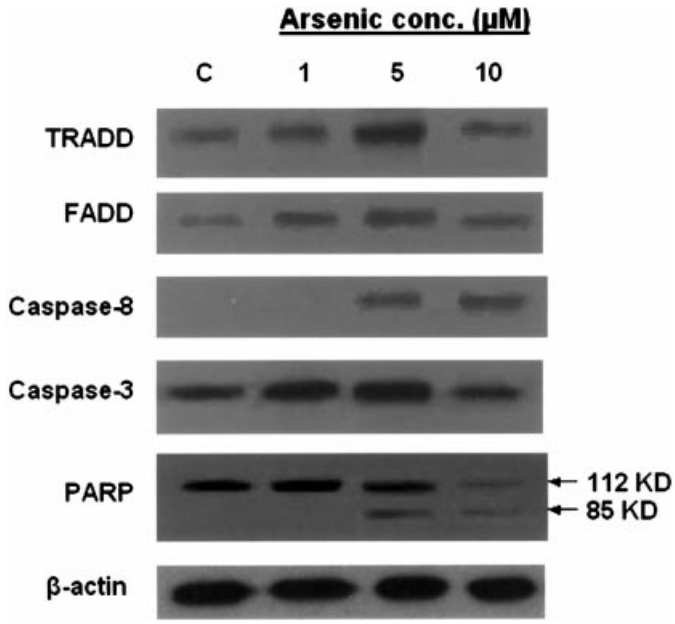

Figure 7. TNF-R1-associated apoptosis signaling factor expression detected by Western blotting. Total cellular proteins were isolated from 48 h arsenic treated or control MNC samples. TRADD, FADD, caspase-8 p20 (active form), caspase-3 p20 (active form), and PARP antibodies were used. PARP antibody can react with the 112 kDa PARP and its 85 kDa fragment specifically cleaved by caspase-3.

signaling complex that initiates apoptosis through activation of caspase-8 (Muzio *et al*, 1996). Caspase-8 in turn induces activation of caspase-3 and caspase-7, which cleave PARP, and of caspase-6, which cleaves lamin B, thus contributing to dissolution of the nuclear envelope (Mizushima *et al*, 1996; Dbaibo *et al*, 1997; Nicholson and Thornberry, 1997; Cuvillier *et al*, 1998; Kimura and Gelmann, 2000). TNF-R1 and TNF-R2 may mediate the activation of independent downstream signaling pathways (Engelmann *et al*, 1990; Tartaglia *et al*, 1991). TNF-R2 functions primarily to bind ligand rapidly, passing soluble TNF trimers to TNF-R1 (Tartaglia *et al*, 1993; Pinckard *et al*, 1997). Engagement by FasL initiates a death signal by causing the aggregation of specific cytoplasmic signaling proteins on the death domain of Fas. Fas receptor interacts with FADD to initiate apoptosis through activation of caspase cascades (Cohen, 1997; Sharma *et al*, 2000). In this study, TNF-R1-related proteins, i.e., TRADD, FADD, caspase-8, caspase-3, and cleavage of PARP were observed. On the other hand, arsenic showed no significant effect on TNF-R2 expression in either CD4+ cells or CD8+ cells. Although arsenic revealed a slightly stimulatory effect on Fas expression in both CD4+ and CD8+ cells, Fas ligand expression could not be detected on either cell. The Fas/FasL system is not considered to be actively involved in arsenic-induced apoptosis. Our results indicate that TNF-R1 signaling plays a significant role in CD4+ cell apoptosis induced by arsenic. The release of TNF-α from MNCs is a reasonable explanation for arsenic-induced CD4+ cell apoptosis. There are two other possible mechanisms for an arsenic-initiated TNF-R1 pathway in CD4+ cells. First, similar to some apoptosis-inducing agents (Madge *et al*, 1999), arsenic may directly react with TNF-R1. Second, arsenic may phosphorylate PEA-15 like proteins (Estelles *et al*, 1999), therefore enhancing apoptosis via the TNF-R1 cell death pathway. More investigation is needed to elucidate the mechanisms, however.

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REFERENCES

Ashkenazi A, Dixit VM: Death receptors: signaling and modulation. *Science* 281:1305–1308, 1998

Brunner T, Mogil RJ, LaFace D, *et al:* Cell-autonomous Fas (CD95) /Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature* 373:441–444, 1995

Chen CJ, Chuang YC, Lin TM, Wu HY: Malignant neoplasms among residents of a blackfood disease-endemic area in Taiwan. High-arsenic artesian well water and cancers. *Cancer Res* 45:5895–5899, 1985

Chen CJ, Hsuch YM, Chiou HY, *et al:* Human carcinogenicity of inorganic arsenic. In: Abernathy CO, Calderon RL, Chappell WR, eds. *Arsenic: Exposure and Health Effects*. London: Chapman & Hall, 1997:pp 232–242

Chen GQ, Zhu J, Shi XG, *et al: In vitro* studies on cellular and molecular mechanisms of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia: As_2O_3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. *Blood* 88:1052–1061, 1996

Cohen GM: Caspases: the executioners of apoptosis. *Biochem J* 326:1–16, 1997

Constant SL, Bottomly K: Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu Rev Immunol* 15:297–322, 1997

Cuvillier O, Rosenthal DS, Smulson ME, Spiegel S: Sphingosine 1-phosphate inhibits activation of caspases that cleave poly (ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. *J Biol Chem* 273:2910–2916, 1998

Dbaibo GS, Perry DK, Gamard CJ, *et al:* Cytokine response modifier A (CrmA) inhibits ceramide formation in response to tumor necrosis factor (TNF): CrmA and Bcl-2 target distinct components in the apoptotic pathway. *J Exp Med* 185:481–490, 1997

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S: A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391:43–50, 1998

Engelmann H, Holtmann H, Brakebusch C, *et al:* Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. *J Biol Chem* 265:14497–14504, 1990

Estelles A, Charlton CA, Blau HM: The phosphoprotein PEA-15 inhibits Fas- but increases TNF-R1-mediated caspase-8 activity and apoptosis. *Dev Biol* 216:16–28, 1999

Hsu H, Shu HB, Pan MG, Goeddel DV: TRADD–TRAF2 and TRADD–FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 84:229–308, 1996

Jha AN, Noditi M, Nilsson R, Natarajan AT: Genotoxic effects of sodium arsenite on human cells. *Mutat Res* 284:215–221, 1992

Jost LM, Kirkwood JM, Whiteside TL: Improved short- and long-term XTT-based colorimetric cellular cytotoxicity assay for melanoma and other tumor cells. *J Immunol Meth* 147:153–165, 1992

Kimura K, Gelmann EP: Tumor necrosis factor-α and Fas activate complementary Fas-associated death-domain-dependent pathways that enhance apoptosis induced by γ-irradiation. *J Biol Chem* 275:8610–8617, 2000

Lee-Chen SF, Yu CT, Jan KY: Effect of arsenite on the DNA repair of UV-irradiated Chinese hamster ovary cells. *Mutagenesis* 7:51–55, 1992

Madge LA, Sierra-Honigmann MR, Pober JS: Apoptosis-inducing agents cause rapid shedding of tumor necrosis factor receptor 1 (TNFR1). *J Biol Chem* 274:13643–13649, 1999

McCabe M, Magvire D, Nowals M: The effects of arsenic compounds on human and bovine lymphocyte mitogeneis *in vitro*. *Environ Res* 31:323–331, 1983

Mizushima N, Koike R, Kohsaka H, Kushi Y, Handa S, Yagita H, Miyasaka N: Ceramide induces apoptosis via CPP32 activation. *FEBS Lett* 395:267–271, 1996

Muzio M, Chinnaiyan AM, Kischkel FC, *et al:* FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/Apo-1) death-inducing signaling complex. *Cell* 85:817–827, 1996

Nagata S: Apoptosis by death factor. *Cell* 88:355–365, 1997

Nicholson D, Thornberry N: Caspases: killer protease. *Trends Biochem Sci* 22:299–306, 1997

Pinckard JK, Sheehan KCF, Schreiber RD: Ligand-induced formation of p55 and p75 tumor necrosis factor receptor heterocomplexes on intact cells. *J Biol Chem* 272:10784–10789, 1997

Roboz GJ, Dias S, Lam G, Lane WJ, Soignet SL, Warrell RP Jr, Rafii S: Arsenic trioxide induces dose- and time-dependent apoptosis of endothelium and may exert an antileukemic effect via inhibition of angiogenesis. *Blood* 96:1525–1530, 2000

Rossman TG: Molecular and genetic toxicology of arsenic. In: Rose J, ed. *Environmental Toxicology: Current Developments*. Amsterdam: Gordon and Breach Science, 1998:pp 207–211

Rossman TG: Arsenite genotoxicity may be mediated by interference with DNA damage-inducible signaling. In: Chappell WR, Abernathy CO, Calderon RL, eds. *Arsenic Exposure and Health Effects*. Amsterdam: Elsevier, 1999:pp 233–241

Rousselot P, Labaume S, Marolleau JP, Larghero J, Noguera MH, Brouet JC, Fermand JP: Arsenic trioxide and melarsoprol induce apoptosis in plasma cell lines and in plasma cells from myeloma patients. *Cancer Res* 59:1041–1048, 1999

Russell JH, White CL, Loh DY, Meleedy-Rey P: Receptor-stimulated death pathway is opened by antigen in mature T cells. *Proc Natl Acad Sci USA* 88:2151–2155, 1991

Sharma K, Wang RX, Zhang LY, *et al:* Death the Fas way: regulation and pathophysiology of CD95 and its ligand. *Pharmacol Ther* 88:333–347, 2000

Shen ZY, Tan LJ, Cai WJ, Shen J, Chen C, Tang XM, Zheng MH: Arsenic trioxide induces apoptosis of oesophageal carcinoma *in vitro*. *Int J Mol Med* 4:33–37, 1999

Smith CA, Farrah T, Goodwin RG: The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death. *Cell* 76:959–966, 1994

Synder RD, Davis GF, Lachmann P: Inhibition by metals of X-ray and ultraviolet-induced DNA repair in human cells. *Biol Trace Element Res* 21:389–398, 1989

Tartaglia LA, Goeddel DV: Two TNF receptors. *Immunol Today* 13:151–153, 1992

Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino MA Jr, Goeddel DV: The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc Natl Acad Sci USA* 88:9292–9296, 1991

Tartaglia LA, Pennica D, Goeddel DV: Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. *J Biol Chem* 268:18542–18548, 1993

Vassalli P: The pathophysiology of tumor necrosis factors. *Annu Rev Immunol* 10:411–452, 1992

Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP: Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol* 17:331–367, 1999

Wang ZG, Rivi R, Delva L, *et al:* Arsenic trioxide and melarsoprol induce programmed cell death in myeloid leukemia cell lines and function in a PML and PML-RARalpha independent manner. *Blood* 92:1497–1504, 1998

Yih LH, Ho IC, Lee TC: Sodium arsenite disturbs mitosis and induces chromosome loss in human fibroblasts. *Cancer Res* 57:5051–5059, 1997

Yu CL, Chang KL, Chiu CC, Chiang BN, Han SH, Wang SR: Alteration of mitogenic responses of mononuclear cells by anti-ds DNA antibodies resembling immune disorders in patients with systemic lupus erythematosus. *Scand J Rheumatol* 18:43–49, 1989

Yu HS, Chang KL, Wang CM, Yu CL: Alterations of mitogenic response of mononuclear cells in arsenical skin cancer. *J Dermatol* 19:710–714, 1992

Yu HS, Chang KL, Yu CL, Wu CS, Chen GS, Ho JC: Defective IL-2 receptor expression in lymphocytes of patients with arsenic-induced Bowen's disease. *Arch Dermatol Res* 290:681–687, 1998

Zhang W, Ohnishi K, Shigeno K, *et al:* The induction of apoptosis and cell cycle arrest by arsenic trioxide in lymphoid neoplasms. *Leukemia* 12:1383–1391, 1998

Zheng J, Deng YP, Lin C, Fu M, Xiao PG, Wu M: Arsenic trioxide induces apoptosis of HPV16 DNA-immortalized human cervical epithelial cells and selectively inhibits viral gene expression. *Int J Cancer* 82:286–292, 1999

Zheng L, Fisher G, Miller RE, Peschon J, Lynch DH, Lenardo MJ: Induction of apoptosis in mature T cells by tumor necrosis factor. *Nature* 377:348–351, 1995